DIACARTA™

PRODUCT INSTRUCTION FOR USE

QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer

CATALOG NUMBER

DC-01-0001R
Pack Size: 96 Reactions

Manufacturer

DiaCarta, Inc.
4385 Hopyard Road, Suite 100
Pleasanton, CA 94588
United States
P: +1 800-246-8878
F: + 1 510-735-8636
E: information@diacarta.com

INTENDED USE

Research Use Only

*Where appropriate, please disregard assay mixes that are not part of your kits.

CONTENTS

PART 1. INTENDED USE

The QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer is a signal amplification nucleic acid probe assay for qualitative detection of human papillomavirus (HPV) E6/E7 mRNA directly from Pap smear samples or liquid based cytology (LBC) specimens without RNA purification or RT-PCR using the DiaCarta Benchtop Luminometer or other 96-well plate luminometers such as SpectraMax L Microplate Reader (Molecular Device Inc.). Detection of HPV high risk subtypes 16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59, 66, and 68 have been validated for the assay. The QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer does not distinguish between the 14 high-risk types when the high-risk probe set is used. HPV16 and HPV18 probes are included in the kit to determine if a HPV positive specimen contains HPV16 and/or HPV18.

The use of the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer is indicated:

- To screen patients with ASC-US (atypical squamous cells of undetermined significance) cervical cytology results to determine the need for referral to colposcopy.
- In women 30 years and older, the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer can be used with cervical cytology to adjunctively screen to assess the presence or absence of high-risk HPV types. This information, together with the physician's assessment of cytology history, other risk factors, and professional guidelines, may be used to guide patient management.
- The results from the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer must be interpreted within the context of all relevant clinical and laboratory findings.

WARNINGS:

- The positive predictive values of HPV RNA are not intended for use in guiding therapy.
- The QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer is not intended for use alone in the diagnosis or confirmation of HPV infection.
- Assay performance characteristics have not been established with individuals who have been vaccinated with HPV vaccines.

PART 2. SUMMARY AND EXPLANATION

The genital human papillomavirus (HPV) infection is the most common sexually transmitted infection. HPV infection has been convincingly proven to be the major risk factor for development of pre-invasive or invasive carcinoma in epidemiologic studies. HPV is transmitted primarily through sexual activities and to some extent by other close personal contact.

Because HPV infections are so widespread, with an estimated 100 million people infected worldwide, early detection is essential to help control the disease. The immune system effectively repels most HPV infections, however, approximately ten percent of individuals develop a persistent infection, with risk of development of high-grade precursor lesions and eventually invasive carcinoma. HPV is a causative factor in the development of cancers of the uterine cervix, which has been firmly established.

HPVs are a large family of small double-stranded DNA virus of approximately 7800 nucleotides which infect squamous epithelia (or cells with the potential for squamous maturation). HPVs are classified by genotype, and at least, about 130 types have been identified by sequences of the gene encoding the major capsid protein L1. HPVs can be classified into high or low-risk types depending upon their oncogenic potentials. High-risk genotypes 16 and 18 are associated with 70% of cervical carcinoma, and about 80% HPV positive vulval and vaginal carcinoma. Low-risk types 6 and 11 have been associated in 90% of genital warts.

There have been recent advances in the understanding of HPV disease. Oncogenic HPV types induce most cancers of the vulva and vagina. In precancerous lesions, most HPV genomes persist in an episomal state whereas, in many high-grade lesions and carcinomas, genomes are found integrated into host chromosome. Two viral genes E6 and E7 are invariably expressed in HPV-positive cancer cells. Their gene products are known to inactivate the major tumor suppressors, p53 and retinoblastoma protein (pRB), that act as inhibitors of apoptosis. Therefore, the presence of oncogenes E6 and E7 have been widely accepted as biomarkers for cervical cancer detection.

Detection of HPV is usually based on pathological screening of liquid based cytology or with Pap smear tissue samples. It is a tedious process with high cost and low specificity. Recently, there have been strong developments in molecular

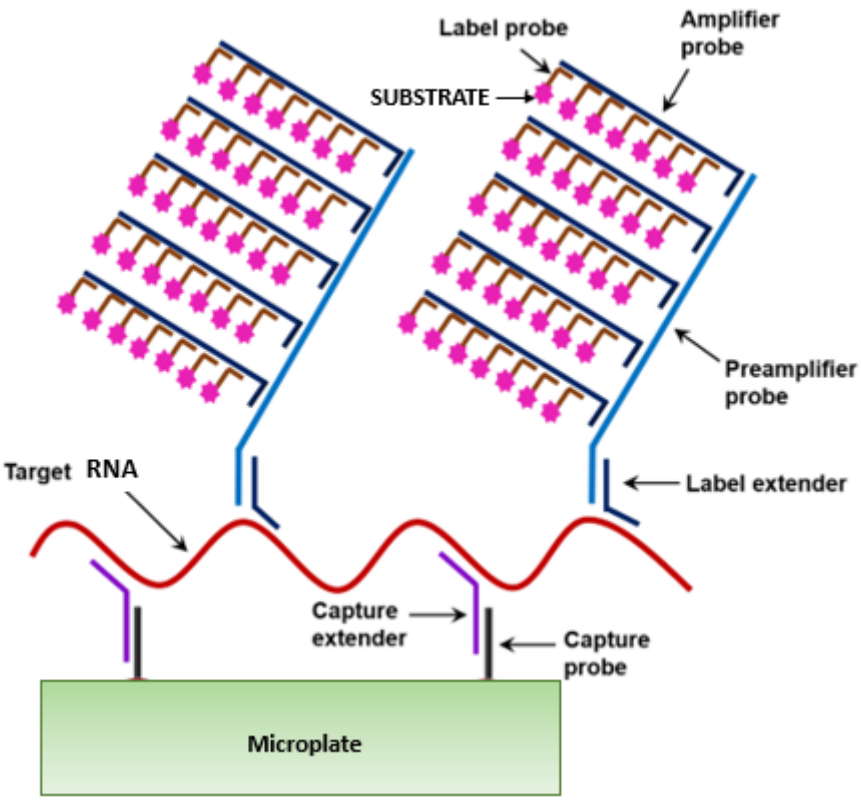
diagnostics for HPV genotyping. However, these tests are all focusing on HPV's L1 regions of DNA, which could be integrated into human genome and generates false negative results.

QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer detects the oncogenes E6/E7 mRNA, which gives much more specific and accurate testing results. Powered by the branched DNA (bDNA) signal amplification technology, the kit provides better sensitivity and specificity than those tests currently available in the market.

PART 3. ASSAY PRINCIPLES

QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer is based on DiaCarta's SuperbDNA™ technology. The SuperbDNA™ technology is a sandwich nucleic acid hybridization procedure to detect HPV E6/E7 mRNA in cervical samples without RNA purification or RT-PCR. After HPV mRNA is released from the cells by lysis, the mRNA is captured onto a microwell by a set of target-specific, synthetic oligonucleotide capture extenders. Another set of target-specific, synthetic oligonucleotides called label extender hybridizes to both the viral mRNA from the HPV genome and the synthetic pre-amplifier probes. The pre-amplifier probe subsequently hybridizes to an amplifier probe forming a branched DNA (bDNA) complex. Multiple copies of an alkaline phosphatase (AP) labeled probe are then hybridized to the immobilized amplifier probe. Detection is achieved by incubating the AP-bound complex with a chemiluminescent substrate. Light emission is directly related to the amount of HPV mRNA present in each sample, and results are recorded as relative light units (RLUs) by the DiaCarta Benchtop Luminometer. A positive control is defined by light emission from control sample containing known concentrations of E6/E7 mRNA. A result of "positive" or "negative" is generated based on relative light units (RLUs) from cervical samples over the RLUs from background.

Figure 1. SuperbDNA™ technology



PART 4. MATERIALS PROVIDED

Materials provided in one kit contain sufficient reagents and materials to perform either one complete or up to three partial plate assays.

IMPORTANT: Upon receiving, store kit components at appropriate storage temperature according to storage temperature in the table 1 and table 2 below.

Component 1

Table 1. Components in DiaCarta Paper Box

Component	Quantity	Part #	Description	Storage
1X Lysis Mixture	1x 30 mL	1000962	Aqueous buffered solution with sodium azide (<0.1%) and other preservatives	2°C to 25°C
Amplifier/Label Probe Diluent	1x 36 mL	1000972	Aqueous buffered solution with a protein-containing preservative	2°C to 25°C
Substrate Enhancer	1x 36 µL	1000982	Aqueous solution with sodium azide (<0.1%) and other preservatives	2°C to 25°C
Plate Seal	15 sheets	1011242	Adhesive-backed foil seal	2°C to 25°C
10X Wash Buffer	1x 50 mL	1001562	Aqueous buffered solution with sodium azide (<0.1%) and other preservatives	2°C to 25°C
1X Sample Wash Solution	1x 25 mL	1007527	Aqueous solution	2°C to 25°C
Capture Plate	1	1001012	Polystyrene microwells coated with synthetic oligonucleotides	2°C to 8°C
Substrate	1x 10.6 mL	1001022	Chemiluminescent substrate	2°C to 8°C

Component 2

Table 2. Components in Pouch

Component	Quantity	Part #	Description	Storage
Proteinase K	1 x 110 µL	1001032	Proteinase K solution	-25°C to-15°C
Blocking Reagent	1 x 110 µL	1001042	Aqueous buffered solution containing a preservative	-25°C to-15°C
HPV High Risk Probe Set	1 x 110 µL	1001052	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
HPV16 Probe set	1 x 110 µL	1001062	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
HPV18 Probe set	1 x 110 µL	1001072	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
Pre-Amplifier Probe	1 x 110 µL	1001082	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
Amplifier Probe	1 x 110 µL	1001092	Synthetic oligonucleotides in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
Label Probe	1 x 110 µL	1001102	Enzyme-labeled Synthetic oligonucleotides in buffered solution with preservatives	-25°C to-15°C
HPV16 Positive Control	1x 330 µL	1001112	Single-stranded DNA in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
HPV18 Positive Control	1x 330 µL	1001122	Single-stranded DNA in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C
High Risk Positive Control	1x 330 µL	1001132	Single-stranded DNA in buffered solution with sodium azide (<0.1%) and other preservatives	-25°C to-15°C

4.1. Shelf life and Multiple Freeze/Thaw Cycles

- The kit has a shelf life of 12 months and reagents should be used within 3 months after package is open.
- The effect of 8 freeze-thaw cycles was tested in kit components stored at -20°C and HPV-positive specimen (HeLa cells spiked in the lysis mixture). There was no effect observed on kit components or HPV-positive specimens mentioned above.
  Caution: Repeated freeze-thaw cycles should be avoided to ensure high and consistent kit performance

4.2. Materials Required but Not Provided

- DiaCarta Benchtop Luminometer with "DiaCarta" bDNA Software (DMS) for the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer, or other equivalent 96-well plate luminometer such as SpectraMax L Microplate Reader (Molecular Device Inc.)
- Incubator, capable of maintaining a constant temperature between 46-55°C ±1°C with forced air circulation
- Adjustable single and multi-channel precision pipettes for dispensing 1-20 µL, 20-200 µL, and 200-1000 µL (accuracy ±5%)
- Pipette tips
- Sterile reagent reservoirs, 25 mL or 50 mL capacities
- 15 mL or 50 mL sterile Nuclease-free polypropylene tubes
- 10 mL sterile-packaged serological pipettes
- 1.5mL microcentrifuge tubes
- Microcentrifuge
- Vortex mixer
- 4-inch soft rubber roller and film-sealing paddle for plate sealing
- Clorox Bleach, unscented (6% sodium hypochlorite)
- 70% Ethanol
- Aluminum foil

4.3. Warnings and Precautions

HARMFUL! IRRITANT! May cause sensitization by inhalation and skin contact. Irritating to eyes, respiratory system and skin. In case of contact with eyes, rinse immediately with plenty of water and seek medical advice. Wear suitable protective clothing. **Contains:** Proteinase K, Lysis Mixture.

IRRITANT! Hazardous in case of skin contact (irritant), of eye contact (irritant). Hazardous in case of ingestion, of inhalation. Inflammation of the eye is characterized by redness, watering, and itching. Skin inflammation is characterized by itching, scaling, reddening, or, occasionally, blistering. Avoid contact with skin and eyes. Avoid formation of dust and aerosols. Wear suitable gloves and clothing. **Contains**: Sodium Azide (CAS# 26628-22-8): Lysis Mixture, Amplifier/Label Probe Diluent, Pre-Amplifier Probes, Amplifier Probe, Label Probe, Wash buffer, and Substrate Enhancer.

CAUTION! POTENTIAL BIOHAZARD:

The positive control contains recombinant viral DNA. Handle as non-capable of transmitting infections agents according to established good laboratory practices and universal precautions.

- Because no test method can offer complete assurance that laboratory specimens do not contain HIV, hepatitis B virus, pathogens involved in sexually transmitted diseases or other infectious agents, specimens should be handled at the BSL 2 as recommended for any potentially infectious human cervical specimen in the CDC-NIH manual, Biosafety in Microbiological and Biomedical Laboratories, 5th Edition, 2009 and CLSI Approved Guideline M29-A3, Protection of Laboratory workers from occupational acquired infections (Third Edition)

- Disposal. Dispose of hazardous or biologically contaminated materials according to the practices of your institution. Discard all materials in a safe and acceptable manner, and in compliance with all federal, state, and local requirements.

CAUTION:

Sodium azide in the reagents can react with copper and lead plumbing to form explosive metal azides. On disposal flush reagents with a large volume of water to prevent the buildup of metal azides, if disposal into a drain is in compliance with federal, state, and local requirements.

Perform the procedure using universal precautions.

- Disinfect spills promptly using a 0.6% sodium hypochlorite solution (1:10 v/v household bleach) or equivalent disinfectant. Handle contaminated materials as biohazardous.
- Wear personal protective apparel, including disposable gloves, throughout the assay procedure. Thoroughly wash hands after removing gloves and dispose of gloves as biohazardous waste.
- Do not eat, drink, smoke, or apply cosmetics in areas where reagents or specimens are handled.
- Avoid the use of sharp objects wherever possible.
- If skin or mucous membrane exposure occurs, immediately wash the area with large amounts of water. Seek medical advice immediately.
- Do not pipette by mouth.
- Use all pipetting devices and instruments with care and follow the manufacturers' instructions for calibration and quality control.
- Use of aerosol-resistant pipette tips are highly recommended and use a new tip every time a volume is dispensed. Aerosol-resistant pipette tips with additives should be avoided.
- Do not use reagents if crystal or precipitate is visible after bringing to specified temperature.
- Do not use components beyond the expiration date printed on the kit boxes.
- Do not mix reagents from different lots.
- Use all kit components within 3 months after opening any component.
- Return all components to the appropriate storage condition after preparing the working reagents.
- For stability of the working reagents, refer to the Assay Procedure section.
- Do not interchange vial or bottle caps as cross-contamination may occur.

PART 5. SPECIMEN COLLECTION AND HANDLING

Individual should follow guidelines for Pap test before specimen collection. Proper specimen handling is very important to protect the RNA from degradation. Use precautions when handling and disposing samples while performing the assay. To avoid cross-contamination, change gloves frequently.

- This assay requires 50 µL of processed sample for a single determination.
- Collect specimens by Pap smear or liquid based cytology methods based on the instructions for the collection device.
- Store specimens at -60° to -80°C in sterile, screw-capped tubes. Specimens may also be stored at 2° to 8°C for up to 48 hours or at -20°C in a non-frost-free freezer for up to 72 hours prior to freezing at -60° to -80°C.
- Avoid repeated freeze/thaw of the samples.
- Handle all specimens as if capable of transmitting infection.
- If necessary, to ship, ship specimens frozen on dry ice. Package and label in compliance with federal and international regulations covering the transport of clinical samples and etiological agents.

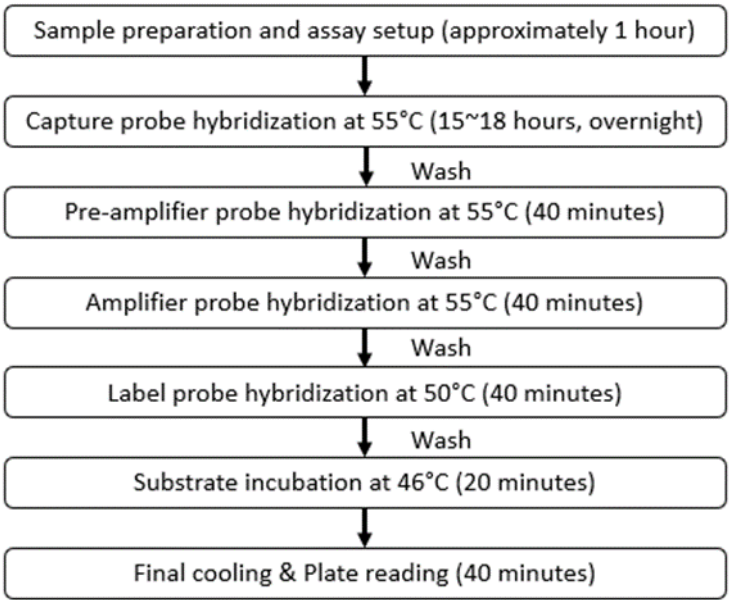
PART 6. ASSAY PROCEDURES

IMPORTANT:

- The procedure consists of two major activities: 1. hybridizing the probes and 2. measuring the light output. The same procedure will be used for the assay for 14 combined high-risk HPV subtype, the assay for subtype 16 and the assay for subtype 18. The only difference is the probe sets used in "Assay Step 2" to capture the target in the samples.
- Before use, make sure to dilute the 10X Wash Buffer with nuclease-free water to obtain 1X Wash buffer concentration. The volume of Wash Buffer provided may be more than the listed volume of 50 mL due to overfilling. The 10X Wash Buffer should be measured before diluting it.

PROCEDURAL NOTES

- The bDNA test is very sensitive to temperature change. The oven temperature should be validated using a digital thermometer.
- Once the plate is removed from the oven, the plate should be processed immediately according to the procedures. Except cooling the plate prior to the reading, any delay is not acceptable since that nonspecific hybridization would occur while the cooling.
- The results are calculated based on the RLUs of samples versus those of Blank.
- Room temperature is 25°C±5°C.
- Clean all pipettors and the benchtop with 70% ethanol before starting the procedure and wear clean gloves.



6.1. Assay Step 1: Lysing the Specimens to Release Viral RNA from Cervical Sample

1) Turn the incubator on setting at 60°C.
2) If there are visible crystals in the Lysis Mixture due to storage at cold temperatures, warm the Lysis Mixture bottle at 37°C until the crystals dissolve. Mix the Lysis Mixture by inverting the bottle several times.
3) Place the Proteinase K on ice and keep chilled.
4) If each cervical sample is stored in 1 mL of Transport Medium, mix well, transfer 0.5 mL (i.e. 50% from the collected specimens) of cervical samples to 1.5 mL centrifuge tubes (make sure to mark the patient ID on each tube) and spin for 5 minutes at 1,000 x g.
5) Discard supernatant and wash the pellet once with 0.5 mL 1x Sample Wash Solution. Spin for 5 minutes at 1,000 x g. Discard the supernatant.
6) For each tissue sample, add 250 µL Lysis Mixture and 2 µL Proteinase K provided.
7) Mix by vortexing for 5 seconds. Incubate at 60°C for 1 hour. Vortex the sample tubes for 5 seconds every 20 min during the incubation to mix.
8) Do a quick spin to ensure pooling of the sample. When using the sample, make sure to pipette the supernatant carefully. Leave on ice until use if using on the same day. Keep the supernatants at -80°C if not used on the same day.

Note: Before loading the sample, sample should be kept on the bench for 10-20 minutes or until any visible crystals dissolve.

6.2. Assay Step 2. Hybridizing the Probe Set with Target RNAs—Capturing Target RNA from Cervical Sample

Note:

- Bring the pouch containing the Capture Plate to room temperature before opening.
- It is recommended to first test the samples with 14 combined high-risk HPV assay, and the positive samples can then be further tested with HPV16 and HPV18 assays for genotyping.
- Two positive controls and three blank controls must be run in every test to evaluate the validity of the assay and for calculation of results for unknown samples.
- It is highly recommended to run two replicates for unknown samples.

1) Turn the incubator on setting at 55°C.
2) If there are visible crystals in the Lysis Mixture due to storage at cold temperatures, warm the Lysis Mixture bottle at 37°C until the crystals dissolve. Mix the Lysis Mixture by inverting the bottle several times.
3) Bring the following reagents to room temperature to thaw.
   - Probe sets for 14 combined High-risk HPV, HPV16 or HPV18 (depending on what assay to run)
   - Vials of High Risk, HPV16 and/or HPV18 Positive Control (depending on what assay to run)
   - The samples to be tested
   - Blocking reagent
4) Calculate the number of reagents needed based on the number of wells, and then prepare Working Probe Solution following the table below (prepare 15% extra) using a 15 ml polypropylene centrifuge tube.

Reagent Name	Volume (µL) Required per Well
Lysis Mixture	48
Blocking Reagent	1
Probe Set *	1

* Use High-risk HPV, HPV16 or HPV18 probe set depending on what assay to run.

5) Gently vortex or invert 10 times to mix the Working Probe Solution.
6) Add 50 µL of Working Probe Solution to each well.
7) Add 50 µL of unknown samples into the appropriate sample well according to the plate map below.
8) Add 50 µL of the appropriate Positive Control to each Positive Control well and 50 µL of Lysis Mixture to each Blank well.

Recommended plate map:

	1	2	3	4	5	6	7	8	9	10	11	12
A	PC											
B	PC											
C	Blank											
D	Blank											
E	Blank											
F												
G												
H												

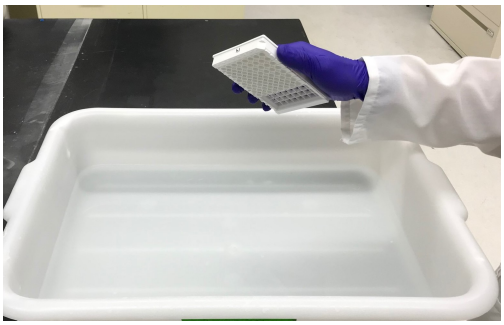
Note:

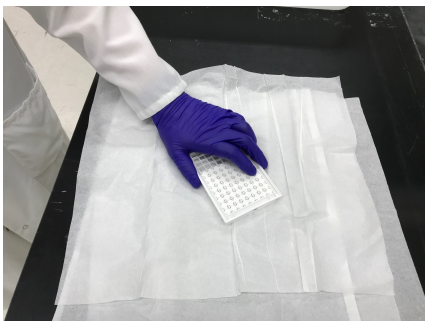
- PC denotes Positive Control; each Positive Control provided in the kit is enough for 6 wells.
- Immediately return remaining specimens to -20°C or -80°C as necessary.

9) Seal the plate tightly with a Plate Seal. Use a soft rubber roller to apply pressure and aid in complete and uniform plate sealing. Use a film-sealing paddle to ensure a tight seal across every individual well and the plate edges. Failure to do so causes potential well-to-well contamination and edge effects due to evaporation.
10) Incubate the plate at 55°C overnight. Make sure the incubator is maintained at 55°C ± 1°C accuracy.

6.3. Assay Step 3: Hybridizing the Pre-Amplifier Probe

1) Bring the Amplifier/Label Probe Diluent to room temperature if necessary.
2) Bring Pre-Amplifier Probe to room temperature until completely thawed.
3) Calculate the amount of Pre-Amplifier Working Reagent to prepare based on the number of wells (make 15% extra). One well needs 100 µL Pre-Amplifier Working Reagent.
4) Prepare Pre-Amplifier Working Reagent in a 15 mL polypropylene tube by 100-fold dilution of Pre-Amplifier Probe with Amplifier/Label Probe Diluent. For example, to prepare 5 mL Pre-Amplifier Working Reagent, add 50 µL Pre-Amplifier Probe to 4.95 mL Amplifier/Label Probe Diluent.
5) Gently vortex or invert 10 times to mix Pre-Amplifier Working Reagent and leave it at room temperature before use.
6) Wash the Capture Plate:
   a. Remove the Capture Plate from the incubator and remove the Plate Seal.
   b. Invert the Capture Plate into an appropriate receptacle (for example, a Biohazard container) and firmly expel the contents (*Left figure below*). Then tap the inverted plate on layers of paper towels to remove residual buffer (*Right figure below*).





   c. Add 260 µL of 1 X Wash Buffer into each well and let soak for 30 seconds.
   d. Repeat step 6b-6c two more times.
   e. Tap the inverted plate on layers of paper towels to remove residual buffer.

7) Remove traces of Wash Buffer
   a. Using force, tap inverted plate on paper towel. Visually make sure there is no residual Wash Buffer left in well. The following steps 7b to 7d are optional if the mentioned equipment is unavailable.
   b. Invert the Capture Plate on 3 laboratory tissue wipes.
   c. Place the inverted plate and tissues in a centrifuge bucket.
   d. Spin at 240 x g for 1 minute. Keep brakes on, if available.
8) Add 100 µL Pre-Amplifier Working Reagent to each well of the Capture Plate.
9) Seal the Capture Plate with a Plate Seal, using a rubber roller and paddle to ensure tight sealing, and incubate at 55°C for 40 min.

6.4. Assay Step 4: Hybridizing the Amplifier Probe

1) Bring the Amplifier/Label Probe Diluent to room temperature if necessary.
2) Bring Amplifier Probe to room temperature until completely thawed.
3) Calculate the amount of Amplifier Working Solution to prepare based on the number of wells (make 15% extra). One well needs 100 µL Amplifier Working Reagent.
4) Prepare Amplifier Working Solution in a 15 mL polypropylene tube by 100-fold dilution of Amplifier Probe with Amplifier/Label Probe Diluent. For example, to prepare 5 mL Amplifier Working Solution, add 50 µL Amplifier Probe to 4.95 mL Amplifier/Label Probe Diluent.
5) Gently vortex or invert 10 times to mix Amplifier Working Solution and leave it at room temperature before use.
6) Wash the Capture Plate:
   a. Remove the Capture Plate from the incubator and remove the Plate Seal.
   b. Invert the Capture Plate into an appropriate receptacle (for example, a Biohazard container) and firmly expel the contents.
   c. Tap the inverted plate on layers of paper towels to remove residual buffer.
   d. Add 260 µL of 1 X Wash Buffer into each well and let soak for 30 seconds.
   e. Repeat step 6b-6c two more times.
   f. Tap the inverted plate on layers of paper towels to remove residual buffer.
7) Remove traces of Wash Buffer
   a. Using force, tap inverted plate on paper towel. Visually make sure there is no residual Wash Buffer left in well. The following steps 7b to 7d are optional if the mentioned equipment is unavailable.
   b. Invert the Capture Plate on 3 laboratory tissue wipes.
   c. Place the inverted plate and tissues in a centrifuge bucket.
   d. Spin at 240 x g for 1 minute. Keep brakes on, if available.
8) Add 100 µL Amplifier Working Reagent to each well of the Capture Plate.
9) Seal the Capture Plate with a Plate Seal, using a rubber roller and paddle to ensure tight sealing, and incubate at 55°C for 40 min.

6.5. Assay Step 5: Hybridizing the Label Probe

1) Bring the Amplifier/Label Probe Diluent to room temperature if not stored at room temperature.
2) Bring Label Probe to room temperature until completely thawed.
3) Calculate the amount of Label Probe Working Solution to prepare based on the number of wells (make 15% extra). One well needs 100 µL Label Probe Working Solution.
4) Prepare Label Probe Working Solution in a 15 mL polypropylene tube by 100-fold dilution of Label Probe with Amplifier/Label Probe Diluent. For example, to prepare 5 mL Label Probe Working Solution, add 50 µL Label Probe to 4.95 mL Amplifier/Label Probe Diluent.
5) Gently vortex or invert 10 times to mix Label Probe Working Solution and leave it at room temperature before use.
6) Wash the Capture Plate:
   a. Remove the Capture Plate from the incubator and remove the Plate Seal.
   b. Invert the Capture Plate into an appropriate receptacle (for example, a Biohazard container) and firmly expel the contents.
   c. Tap the inverted plate on layers of paper towels to remove residual buffer.
   d. Add 260 µL of 1 X Wash Buffer into each well and let soak for 30 seconds.
   e. Repeat step 6b-6c two more times.
   f. Tap the inverted plate on layers of paper towels to remove residual buffer.

7) Remove traces of Wash Buffer
   a. Using force, tap inverted plate on paper towel. Visually make sure there is no residual Wash Buffer left in well. The following steps 7b to 7d are optional if the mentioned equipment is unavailable.
   b. Invert the Capture Plate on 3 laboratory tissue wipes.
   c. Place the inverted plate and tissues in a centrifuge bucket.
   d. Spin at 240 x g for 1 minute. Keep brakes on, if available.
8) Add 100 µL Label Working Reagent to each well of the Capture Plate.
9) Seal the Capture Plate with a Plate Seal, using a rubber roller and paddle to ensure tight sealing, and incubate at 50°C for 40 min.

6.6. Assay Step 6: Adding Substrate

1) Place the Substrate Enhancer and Substrate on the bench top and bring to room temperature.
2) Calculate the amount of Substrate Working Solution to prepare based on the number of wells (make 15% extra). One well needs 100 µL Substrate Working Solution.
3) Prepare the Substrate Working Solution in a 15 mL polypropylene tube by adding Substrate Enhancer to Substrate at 1:333 ratio. For example, to prepare 5 mL Substrate Working Solution, add 15 µL Substrate Enhancer to 5 mL Substrate.
4) Gently vortex or invert 10 times to mix.
   Note: It is normal for the Substrate Working Solution to become turbid after the addition of Substrate Enhancer.
5) Store Substrate Working Solution at room temperature in the dark before use. The solution can be wrapped in aluminum foil to minimize exposure to light.
6) Wash the Capture Plate:
   a. Remove the Capture Plate from the incubator and remove the Plate Seal.
   b. Invert the Capture Plate into an appropriate receptacle (for example, a Biohazard container) and firmly expel the contents.
   c. Tap the inverted plate on layers of paper towels to remove residual buffer.
   d. Add 260 µL of 1 X Wash Buffer into each well and let soak for 30 seconds.
   e. Repeat step 6b-6c two more times.
   f. Tap the inverted plate on layers of paper towels to remove residual buffer.
7) Remove traces of Wash Buffer
   a. Using force, tap inverted plate on paper towel. Visually make sure there is no residual Wash Buffer left in well. The following steps 7b to 7d are optional if the mentioned equipment is unavailable.
   b. Invert the Capture Plate on 3 laboratory tissue wipes.
   c. Place the inverted plate and tissues in a centrifuge bucket.
   d. Spin at 240 x g for 1 minute. Keep brakes on, if available.
8) Add 100 µL Substrate Working Solution to each well of the Capture Plate.
9) Seal the Capture Plate with a Plate Seal, using a rubber roller and paddle to ensure tight sealing, and incubate at 46°C for 20 min.
10) Remove the plate from the incubator and let it cool for 30 minutes at room temperature without removing the plate seal. Remove the plate seal and read the plate immediately after the cooling period.

6.7. Assay Step 7: Luminescence Measurement and Data Analysis

Follow the procedure below to read the plate on DiaCarta Benchtop Luminometer:

1) Get the DiaCarta Benchtop Luminometer ready.
2) During the substrate incubation, prepare the DMS DiaCarta Benchtop Luminometer software to receive the data from the DiaCarta Benchtop Luminometer. Refer to Analyzing Samples in the operator's manual for more information.
3) Ensure that all maintenance has been performed. If required, update the maintenance log.
4) Turn on the machine.
5) Open DiaCarta Benchtop Luminometer software.
6) Fill in patient sample information according to each test well.
7) Appoint the wells with Positive controls according to plate map.
8) Appoint the wells as Blank for background according to plate map.
9) Save the template.
10) When the plate is ready to read. Select Run.
11) Select print the report after data have been collected.

12) The DiaCarta Benchtop Luminometer automatically reads the light units in each of the wells of the plates and transfers the data to the DMS DiaCarta for analysis.

Follow the procedure below to read the plate on SpectraMax L Microplate Reader (Molecular Device Inc.):

1) Open SoftMax Pro software and connect to SpectraMax L Microplate Reader.
2) Click on Acquisition button and select appropriate parameters as below:
   - Read Mode: Lum
   - Read Type: Endpoint
   - Number of Wavelength: 1
   - Plate Type: 96 wells standard opaque
   - Read Area: select the desired wells
   - PMT gain: automatic
   - Shake: unselect “Before first read” (NO shaking required)
3) Open the drawer and load the plate.
4) Close the drawer and start the run.
5) Export the results for analysis.

6.8. Evaluating the Validity of Assay

To monitor assay performance, two replicates of positive control material and three blanks must be included with every assay. Treat all control samples the same as specimens.

The assay is considered valid if both replicates of Positive Control Signal Ratio (RLU of Positive Control/Average RLU of Blank) is above 20, and the coefficient of variation (CV%) of RLUs from Blank is less or equal to 25%.

If the assay must be repeated, then do the following:

- Review these instructions to ensure that the assay is performed according to the procedures.
- Assure proper placement of positive controls and blank according to the plate map.
- Verify that the materials are not expired.
- Verify that the component lot numbers are matched to the appropriate kit lot. Refer to the product insert supplement.
- Verify that the required maintenance was performed for the DiaCarta Benchtop Luminometer or the oven temperature is calibrated for manually performed assay.

PART 7. CALCULATION OF RESULTS

7.1. Test Results Interpretation

- Calculate Test sample RLU ratio using the formula below:
  Test sample RLU ratio = RLU_{sample} / (1.5 x Average RLU_{Blank})

- If Test sample RLU ratio is equal to or higher than 1.0 (≥1.0), the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer result for the patient is positive. Otherwise, the result is negative.

- When testing samples in duplicate, if one replicate shows positive and the other is negative, then the sample should be re-tested.

- Grey zone: 0.8 ~ 1.0. Recommend repeating the test for samples falling within the grey zone.

False Negative: If RNA/DNA from samples are not enough, then reaction would not work well. Or, samples might contain interference that inhibit the reaction.

False Positive: Other interferences might show background noise then cause false positive results.

Note: The QuantiVirus™ HPV E6/E7 mRNA Test has been validated on both DiaCarta Benchtop Luminometer and SpectraMax L Microplate Reader (Molecular Device Inc.). When using other luminometers, please contact DiaCarta for validation instructions.

7.2. Limitations

Use of this product is limited to personnel trained in bDNA testing.

Reliable results are dependent on correctly following the procedure in this manual.

PART 8. PERFORMANCE CHARACTERISTICS: NON-CLINICAL STUDIES

8.1. Limit of Detection (LoD)

The Limit of Detection (LoD) is the lowest concentration of virus that yields an assay result at or above the detection cutoff 95% of the time. The LoD was determined by examining the detection rate of 10 replicates of low concentration of ssDNA or HeLa cells and the 95% detection limit was calculated from Probit analysis.

Table 3. Limit of Detection in QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer

Template	Probe	Limit of Detection (95%)
HeLa cell lysate	High Risk (14)	2 cells
HeLa cell lysate	HPV 18	2 cells
HPV 18 ssDNA	HPV 18	1,960 copies
HPV 16 ssDNA	HPV 16	3,538 copies

8.2. Reproducibility

To establish the reproducibility of QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer, a precision panel was created with defined analyte levels of HeLa cells (0-1000 cells/well). Each analyte level was tested in triplicates to obtain standard deviation. Two different reagent lots were tested as well. The CV results are listed in Table 4.

Table 4. Reproducibility result summary

Assay type	Percent CV's
Intra-assay	≤10%
Inter-assay	≤ 15%
Lot to Lot variation	≤ 20%

8.3. Potentially Interfering Exogenous Substances

The potential interference of pathogens that may be found in cervical specimens was tested by processing these pathogens as if they were specimens and tested in Calibri (Body). The pathogens tested were listed in Table 5. The pathogens listed in Table 5 were found to have no effect on the QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer at the indicated concentrations. The presence of the non-targeted HPV genotypes tested at the indicated concentration does not present false positive result when tested in QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer. This result indicates that QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer is specific to detect targeted HPV, not to other related but non-targeted HPV genotypes also commonly found in cervical samples.

The potential interference of commonly prescribed drugs and commercial products that may be found in specimens was tested by individually spiking these substances to HPV -negative specimens in the presence or absence of HPV infected cultured cells (6 cells/well). Potentially interfering substance are listed in the table 6. Interference was observed with one of the three anti-fungal medications that contained tioconazole.

Table 5. Potentially interfering pathogens tested

Pathogens Test (Strain)	Test concentration with No Cross-Reactivity
Hepatitis B Virus	5,000 IU/mL
Hepatitis C Virus	25,000 IU/mL
Cytomegalovirus (AD-169)	25,000 cp/mL
Human Immunodeficient Virus-1 (IIIB)	25,000 IU/mL
Herpes Simplex Virus 1 (MacIntyre)	25,000 cp/ml
Herpes Simplex Virus 2 (MS)	25,000 cp/ml
Epstein-Barr Virus (B95-8)	25,000 cp/mL
Influenza A H1N1 (A/NY/02/2009 H1N1)	Qualitative
Influenza B Virus (B/Panama/45/90)	Qualitative
Clostridium difficile (NAP1)	Qualitative
Neisseria gonorrhoeae (Z017)	Qualitative
Staphylococcus aureus (MRSA;COL)	Qualitative
Escherichia coli (O157:H7;EDL933)	Qualitative
Chlamydia trachomatis (D-UW3)	Qualitative
Non-Targeted Human Papilloma Virus	
HPV6-b	165,680 copies per well
HPV 11	165,723 copies per well
HPV 40	165,465 copies per well
HPV 42	163,642 copies per well
HPV 43	165,594 copies per well
HPV 44	171,970 copies per well

Table 6. Potentially Interfering Substance Tested in QuantiVirus™ HPV E6/E7 mRNA Test for Cervical Cancer

Category	Product Brand or Type	Highest Concentration found not interfering with HPV assay
Lubricant	Target Brand personal lubricant liquid (comparable to KY natural feeling)	5% v/v
	Astroglide natural personal lubricant	5% v/v
	Poise Personal Lubricant	10% v/v
	KY Warming Jelly personal lubricant	5% v/v
Anti-fungal Medication	Target Brand Vagicaine Cream	10% w/v
	Target Brand Miconzole 3	5% w/v
Spermicide	Gynol II Vaginal contraceptive gel Extra	10% w/v
	VCF Vaginal Contraceptive Foam	5% w/v
	Conceptrol Vaginal Contraceptive gel	10% w/v
Douche	Target Brand Feminine douche	10% v/v
	Poise Daily Freshness Famine Wash	10% v/v
	Summer's Eve Cleaning wash for sensitive skin	10% v/v
Whole blood	Female Whole Blood	10% v/v

PART 9. TROUBLESHOOTING

9.1. Poor Detection Signal

Possible Cause	Recommended Solutions
The amount of target in specimen is lower than detection limit	Increase specimen input.
Hybridization temperature is not correct	Calibrate the incubator to maintain a constant and even temperature and make sure the setting for each step is correct.
Skip steps or reagents were added in wrong order	Exactly follow the manual for each step. Use Amp/Label Probe Diluent to dilute pre-Amplifier, Amplifier, and Label Probe.
Reagents expired	Reagents should be used within 3 months after package is open.
Alkaline Phosphatase is inactivated	The incubation temperature cannot be over 50°C for Label Probe hybridization.
Wells were dried at some point	Once the signal amplification starts, the wells shouldn't be exposed to air for more than 5 min to dry out.
Reagents especially probes were frozen and thawed more than recommended use	Only thaw reagents within the recommended freeze-thaw cycle.

9.2. High Background

Possible Cause	Recommended Solutions
Residual Wash Buffer	Check carefully that the plate wash method completely removes all residual Wash Buffer before moving to the next step.
Hybridization temperature is not correct	Calibrate the incubator to maintain a constant and even temperature and make sure the setting for each step is correct.
Reagents expired	Reagents should be used within 3 months after package is open.
Capture plate sat at room temperature longer than 20 min after the addition of sample	Do not let the capture plate sit at room temperature for longer than 20 minutes after the addition of the overnight hybridization mixture.
Capture plate sat at room temperature longer than 10 minutes before washing	Wash the capture plate within 10 minutes after removal from the incubator.
Filter tips have additives	We highly recommend not use filter tips with additives.
Contamination of reaction	Use clean gloves every time; Use filter tip and sterile pipet.
Evaporation	Seal the plate tightly according to the instructions.

9.3. Non-Uniform Signal Across the Plate

Possible Cause	Recommended Solutions
Temperature Variation in the Incubator	Verify that the incubator maintains a constant, even temperature.
Temperature variation on the capture plate at time of reading	Read plate 30 min after being taken out of 46°C oven and make sure the whole plate is cooled down.
Incomplete sealing during the incubation	Only use supplied plate seal. Make sure the edges of wells are clearly visible from under the foil seal.
Capture plates were exposed to moisture prior to the assay	Allow the capture plate to come to room temperature for 30 minutes before opening the sealed foil pouch to avoid condensation.
Salt concentrations are variable	Hybridization is affected by salt concentration. When diluting samples, always used the appropriate diluent. Warm Lysis mixture at 37°C to make sure no crystals were present when making working probe solution.

9.4. Well-to-Well Variation

Possible Cause	Recommended Solutions
Inaccurate pipetting	Calibrate the pipettes to make sure the accuracy is ±5%.
Temperature variation in the incubator	Calibrate the incubator to maintain a constant and even temperature.
Residual wash buffer	Make sure the plate wash method used completely removes all residual wash buffer before moving to the next step in the procedure.
Introducing bubbles into wells	Only pour reagents into reservoir without bubbles. Try to pipet with no bubbles.
Insufficient mixing of reagents	Mix the reagents well by inverting the tubes several times. Mix the reagents again before transferring to reagent reservoir.
Scratch of the capture well surface	Minimize contact with the capture plate well surfaces during all addition and washing steps.
Crosstalk among neighboring wells during reading	Only use luminometer with crosstalk < 0.001%.
Salt concentration	Hybridization is affected by salt. When diluting samples, always use the appropriate diluent.
Non-homogenous specimens	Warm specimens to 37°C to dissolve any precipitates and vortex briefly before use.
Specimens too viscous to pipet accurately	Dilute specimens 1:2 in the appropriate diluent before use.

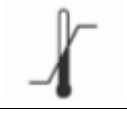
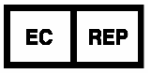
9.5. Day-to-Day Variation

Possible Cause	Recommended Solutions
Temperature variation in the incubator	Calibrate the incubator to maintain a constant and even temperature.
Incubation time varies	Keep incubation duration consistent, especially for incubation with substrate and cool down time.
Non-constant time between substrate incubation ends and plate read	Make sure that time between taking the plate out of the oven after substrate incubation ends and plate read is consistent.

9.6. Potential Contamination

Possible Cause	Recommended Solutions
Contamination during washing process	Do Not blot on area that has been previously used as cross-contamination could occur.
Aerosol contamination in the air	Avoid aerosol in the air. Make sure the lab is clean and free of dust. Observe the wells in the process to see if there are any visible particles falling into the wells.
Powdered gloves or infrequent change of gloves	Use powder-free gloves to avoid introducing unknown powder into reaction wells.

PART 10. SYMBOLS USED IN PACKAGING

Symbol	Definition
	Catalog Number
	Manufactured By
	Temperature Limitation
	Batch Code
	Expiration Date
	Authorized Representative in the European Community
	Research Use only
2012-11-25	Date Format (year-month-day)
2012-11	Date Format (year-month)

REFERENCES

1. Muñoz, N.; Castellsagué, X.; de González, AB. & Gissmann, L. (2006). Chapter 1: HPV in the etiology of human cancer. Vaccine, Aug 31, Vol.24, Suppl.3, S3/1-10, PMID: 16949995
2. McCredie, MR.; Sharples, KJ.; Paul, C.; Baranyai, J.; Medley, G.; Jones, RW. & Skegg, DC. (2008). Natural history of cervical neoplasia and risk of invasive cancer in women with cervical intraepithelial neoplasia 3: a retrospective cohort study. Lancet Oncol, May, Vol.9, No.5, pp. 425-34, PMID: 18407790
3. Stanley, M. (2006). Immune responses to human papillomavirus. Vaccine, Mar 30, Vol.24, Suppl.1, S16-22, PMID: 16219398
4. zur Hausen H. (1996). Papillomavirus infections-a major cause of human cancers. Biochem Biophys Acta, Oct 9, Vol.1288, No.2, F55-78, PMID: 8876633
5. zur Hausen, H. (1999). Papillomaviruses in human cancers. Proc Assoc Am Physicians, Nov-Dec, Vol.111, No.6, pp. 581-7, PMID: 10591087
6. de Villiers, EM.; Fauquet, C.; Broker TR.; Bernard HU. & zur Hausen H. (2004). Classification of papillomaviruses. Virology, Jun 20, Vol.324, No.1, pp. 17-27, PMID: 15183049
7. Bravo, IG.; de Sanjosé, S. & Gottschling, M. (2010). The clinical importance of understanding the evolution of papillomaviruses. Trends Microbiol, Oct, Vol.18, No.10, pp. 432-8, PMID: 20739182
8. Van Doorslaer, K.; Bernard, HU.; Chen, Z.; de Villiers, EM.; Hausen, HZ. & Burk, RD. (2011). Papillomaviruses: evolution, Linnaean taxonomy and current nomenclature. Trends Microbiol, Feb, Vol.19, No.2, pp. 49-50, PMID: 21144754
9. Muñoz, N.; Bosch, FX.; de Sanjosé, S.; Herrero, R.; Castellsagué, X.; Shah, KV.; Snijders, PJ. & Meijer, CJ. (2003). International Agency for Research on Cancer Multicenter Cervical Cancer Study Group. Epidemiologic classification of human papillomavirus types associated with cervical cancer. N Engl J Med, Feb 6, Vol.348, No.6, pp. 518-27, PMID: 12571259
10. Madeleleine, MM.; Daling, JR.; Carter, JJ.; Wipf, GC., Schwartz, SM.; McKnight, B; Kurman, RJ.; Beckmann, AM.; Hagensee, ME. & Galloway, DA. (1977). Cofactors with human papillomavirus in a population-based study of vulvar cancer. J Natl Cancer Inst, Oct 15, Vol. 89, No. 20, pp. 1516-023, PMID: 9337348
11. Daling, JR.; Madeleine, MM.; Schwartz, SM.; Shera, KA; Carter, JJ.; McKnight, B.; Porter, PL.; Galloway, DA.; McDougall, JK. & Tamimi, H. (2002). A population-based study of squamous cell vaginal cancer: HPV and cofactors. Gynecol Oncol, Feb, Vol.84, No.2, pp. 263-70, PMID: 11812085
12. Hampl, M.; Sarajuuri, H.; Wentzensen, N.; Bender, HG. & Kueppers, V. (2006). Effect of human papillomavirus vaccines on vulvar, vaginal, and anal intraepithelial lesions and vulvar cancer. Obstet Gynecol, Dec, Vol.108, No.6, pp. 1361-8, PMID: 17138767
13. Aubin, F.; Prétet, JL.; Jacquard, AC.; Saunier, M.; Carcopino, X.; Jaroud, F.; Pradat, P.; Soubeyrand, B.; Leocmach, Y.; Mougin, C. & Riethmuller, D. (2008). EDiTH Study Group. Human papillomavirus genotype distribution in external acuminata condylomata: a Large French National Study (EDiTH IV). Clin Infect Dis, Sep 1, Vol.47, No. 5, pp. 610-5, PMID: 18637758
14. Münger, K. & Phelps WC. (1993). The human papillomavirus E7 protein as a transforming and transactivating factor. Biochim Biophys Acta, May 25, Vol.1155, No.1, pp. 111-23, PMID: 8389201
15. Ishiji, T. (2000). Molecular mechanism of carcinogenesis by human papillomavirus-16. J Dermatol, Feb, Vol.27, No.2, pp. 73-86, PMID: 10721654